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The *IL17F* and *IL17RA* Genetic Variants Increase Risk of Cerebral Malaria in Two African Populations

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Cerebral malaria (CM) is a neurological complication of infection with *Plasmodium falciparum* that is partly caused by cytokine-mediated inflammation. It is not known whether interleukin-17 (IL-17) cytokines, which regulate inflammation, control the development of CM. To evaluate the involvement of IL-17 cytokines in CM, we analyzed 46 common polymorphisms in *IL17A*, *IL17F*, and *IL17RA* (which encodes the common receptor chain of the members of the IL-17 family) in two independent African populations. A case-control study involving 115 Nigerian children with CM and 160 controls from the community (CC) showed that *IL17F* reference single nucleotide polymorphism (SNP) 6913472 (rs6913472) ($P = 0.004$; odds ratio [OR] = 3.12), *IL17F* rs4715291 ($P = 0.004$; OR = 2.82), *IL17RA* rs12159217 ($P = 0.01$; OR = 2.27), and *IL17RA* rs41396547 ($P = 0.026$; OR = 3.15) were independently associated with CM. A replication study was performed in 240 nuclear Malian family trios (two parents with one CM child). We replicated the association for 3 SNPs, *IL17F* rs6913472 ($P = 0.03$; OR = 1.39), *IL17RA* rs12159217 ($P = 0.01$; OR = 1.52), and *IL17RA* rs41396547 ($P = 0.04$; OR = 3.50). We also found that one additional SNP, *IL17RA* rs41433045, in linkage disequilibrium (LD) with rs41396547, was associated with CM in both Nigeria and Mali ($P = 0.002$; OR = 4.12 in the combined sample). We excluded the possibility that SNPs outside *IL17F* and *IL17RA*, in strong LD with the associated SNPs, could account for the observed associations. Furthermore, the results of a functional study indicated that the aggravating GA genotype of *IL17F* rs6913472 was associated with lower IL-17F concentrations. Our findings show for the first time that *IL17F* and *IL17RA* polymorphisms modulate susceptibility to CM and provide evidence that IL-17F protects against CM.

Cerebral malaria (CM) is one of the most severe complications of infection with *Plasmodium falciparum* and occurs predominantly in young children under 5 years of age and in "nonimmune" adults. The clinical characteristics of CM are an unarousable coma lasting for at least 1 h, with or without generalized convulsions, and asexual *P. falciparum* parasitemia with normal cerebrospinal fluid and no other cause of encephalopathy. This reversible encephalopathy is characterized by the sequestration of infected red blood cells (IRBC) in the capillaries of the brain together with the accumulation of leukocytes, platelets, and uninfected red blood cells (URBC) causing mechanical obstruction of microvessels and excessive activation of immune cells. Other pathological consequences are brain edema, alterations of the integrity of the blood-brain barrier (BBB), microhemorrhages, and tissue necrosis (1). However, despite the large number of studies that have investigated CM, the orchestration of the pathogenic mechanisms leading to CM is not well understood.

Proinflammatory cytokines are thought to contribute to brain pathology in CM. Interleukin-17A (IL-17A) and IL-17F, the best-studied members of the IL-17 family (2, 3), control infections at epithelial barriers (4, 5); they were also shown to aggravate autoimmune diseases and inflammation, including in the brain (5–7). We tested here the hypothesis that IL-17A and IL-17F could aggravate CM in children. We performed in samples from two African populations a study of association between CM and common genetic variants in *IL17A*, *IL17F*, and *IL17RA* (encoding the chain common to all receptors of the IL-17), providing comprehensive coverage of these genes. Our data indicate that genetic variants in *IL17F* and *IL17RA* are associated with susceptibility to CM in the two study populations. Furthermore, our findings provide evidence that IL-17F protects against CM.

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TABLE 1 Demographics of genetic study participants[a]

Parameter	Result(s)			
	Discovery cohort (city of Ibadan, Nigeria)			Replication cohort (city of Bamako, Mali)
Study design	Cases	Controls	Uncomplicated	Family based
Total no. of participants	115	160	89	240 trios
Mean age (range)	4.5 yrs (10 mo–13 yrs)	6 yrs (6 mo–13 yrs)	4.5 yrs (6 mo–13 yrs)	6 yrs (10 mo–15 yrs)
No. (%) of females	55 (47.8)	77 (48.1)	39 (43.8)	119 (49.6)
No. with indicated coma score (Blantyre)	≤2	5	5	≤2
No. (%) with hematocrit:				
>15%	112 (97.4)	160 (100)	89 (100)	210 (87.5)
≤15%	3 (2.6)			30 (12.5)

[a] Severe cases ("Cases") were defined as consisting of children with cerebral malaria (CM, $n = 112$) and as cerebral malaria with severe malarial anemia (CMSMA, $n = 3$). Controls were defined as consisting of community controls (CC, $n = 160$). Uncomplicated malaria (UM, $n = 89$) cases were defined as consisting of febrile children with *P. falciparum* parasitemia.

MATERIALS AND METHODS

Study participants. The discovery cohort was recruited among children from Ibadan, Nigeria. This city has the second largest urban population in the Yoruba plateau, which is a region of holoendemic transmission. We used the Nigerian sample as the discovery cohort because the linkage disequilibrium (LD) map of the Yoruba is described as an African reference population in data banks. The replication cohort was recruited among children from Bamako (Mali) who were living under conditions of seasonal malaria.

The internationally recognized joint ethics committee of the College of Medicine of the University of Ibadan and the University College Hospital in Ibadan approved the Nigerian case-control study. Parents or guardians of children from the city of Ibadan gave written informed consent for their children to participate in the study. The Malian study was approved by the local ethic committees of the Faculty of Medicine of the University of Bamako; written informed consent was obtained from all parents.

All participating children from Ibadan were recruited under the direction of the Childhood Malaria Research Group (CMRG) at the Department of Pediatrics of the University College Hospital (UCH), Ibadan, Nigeria, as previously described (8–11). Briefly, children were 6 months to 13 years of age. WHO criteria were used to define severe malaria (12, 13). CM was defined as a state of unarousable coma (Blantyre coma score ≤ 2) lasting for at least 1 h accompanied by asexual *Plasmodium falciparum* parasitemia with normal cerebrospinal fluid results. Children with CM were also considered to have severe malarial anemia (SMA) if they had a packed cell volume (PCV) of less than 16%. The group of controls from the community (CC) included age-matched parasite-negative healthy children from the same community. Uncomplicated malaria (UM) cases were defined as febrile children with *P. falciparum* parasitemia and with a PCV greater than 20% who did not require hospital admission.

Malian children with CM were recruited as described previously (14–17). The children with CM were hospitalized between 1999 and 2003 in the pediatric department of the Gabriel Toure Hospital in Bamako (Mali). A total of 240 trios (two parents with one CM child) were recruited for the family-based association studies. All these nuclear families were prospectively recruited. The criteria used to define children with a CM phenotype were a coma with a Blantyre score of ≤2 and a thick blood smear positive for *P. falciparum*. Meningitis was ruled out by lumbar puncture. The two study populations are described in Table 1.

Preparation of genomic DNA and selection of single nucleotide polymorphisms (SNPs) for analysis. Genomic DNA from the Nigerian cohort was extracted from peripheral blood leukocytes with a QIAamp blood kit (Qiagen). Genomic DNA from the Malian cohort was extracted as described previously (14).

For the discovery stage, SNPs in the Nigerian samples with a minor allele frequency (MAF) of ≥0.05 were selected within *IL17A*, *IL17F*, and *IL17RA* genes from the 1000 Genomes Yoruba (YRI) database (18).

PLINK (19) was used to determine correlation bins ($R^2 \geq 0.8$) and TagSNPs (i.e., SNPs in a region of the genome with high linkage disequilibrium that represent a group of SNPs) within 5 kb upstream and downstream from the genes. We selected one SNP per correlation bin and a few singletons, providing comprehensive coverage of these genes ($n = 48$; see Table S1 in the supplemental material). For the replication stage in the Malian samples, SNPs associated with CM in the Nigerian samples ($P < 0.05$) were analyzed ($n = 4$). When the SNP was replicated, we also included additional SNPs that were in linkage disequilibrium (LD) with them ($R^2 > 0.6$ [assessed by PLINK using the 1000 genome YRI database]) (18) and located 500 kb upstream or downstream from the associated SNP ($n = 21$, Fig. 1).

Genotyping and quality control. For the discovery stage, genotyping was performed with 115 cases and 160 controls of the Nigerian sample using the custom designed Sequenom IPLEX assay. Genotyping was performed following the manufacturer's instructions. Two SNPs (reference SNP 12201582 [rs12201582] and rs115866730 of *IL17F*) had a low (<90%) call rate and were excluded from the analysis (see Table S1 in the supplemental material). Four SNPs associated with CM and 21 SNPs in LD with them (Fig. 1) were genotyped in the replication Malian sample using either the IPLEX assay (Sequenom) or the TaqMan SNP genotyping assays (Applied Biosystems). SNPs rs112979228 and rs41426844 in *IL17RA* were not informative and were excluded from the study.

Plasma collection and cytokine assays. Plasma samples were collected from CM and UM children from Mali. For UM, the subjects had a thick blood film positive for *P. falciparum*, a Blantyre score of >4, and a hematocrit level of >21%. These children had never developed CM. They attended the outpatient clinic for an episode of febrile malaria. For CM, the criteria described previously were used.

IL-17A and IL-17F concentrations were determined by enzyme-linked immunosorbent assays (ELISAs) with pairs of cytokine-specific monoclonal antibodies according to the manufacturer's instructions. The detection thresholds were 8 pg/ml for IL-17A (R&D) and 15.6 pg/ml for IL-17F (eBiosciences).

Cell cultures and functional study. Peripheral blood mononuclear cells (PBMCs) were isolated by blood centrifugation on Ficoll-Paque (GE Healthcare) (400 × *g*, 45 min), were washed two times in phosphate-buffered saline (PBS)–2 mM EDTA, and were resuspended in supplemented medium. We cultured 1×10^6 cells per well per 100-μl volume in a 24-well microplate in the presence of anti-CD3/CD28 antibodies or in the presence of medium alone. Plates were incubated at 37°C in a 5% CO_2 atmosphere. Supernatants were collected at 3 days, centrifuged, and

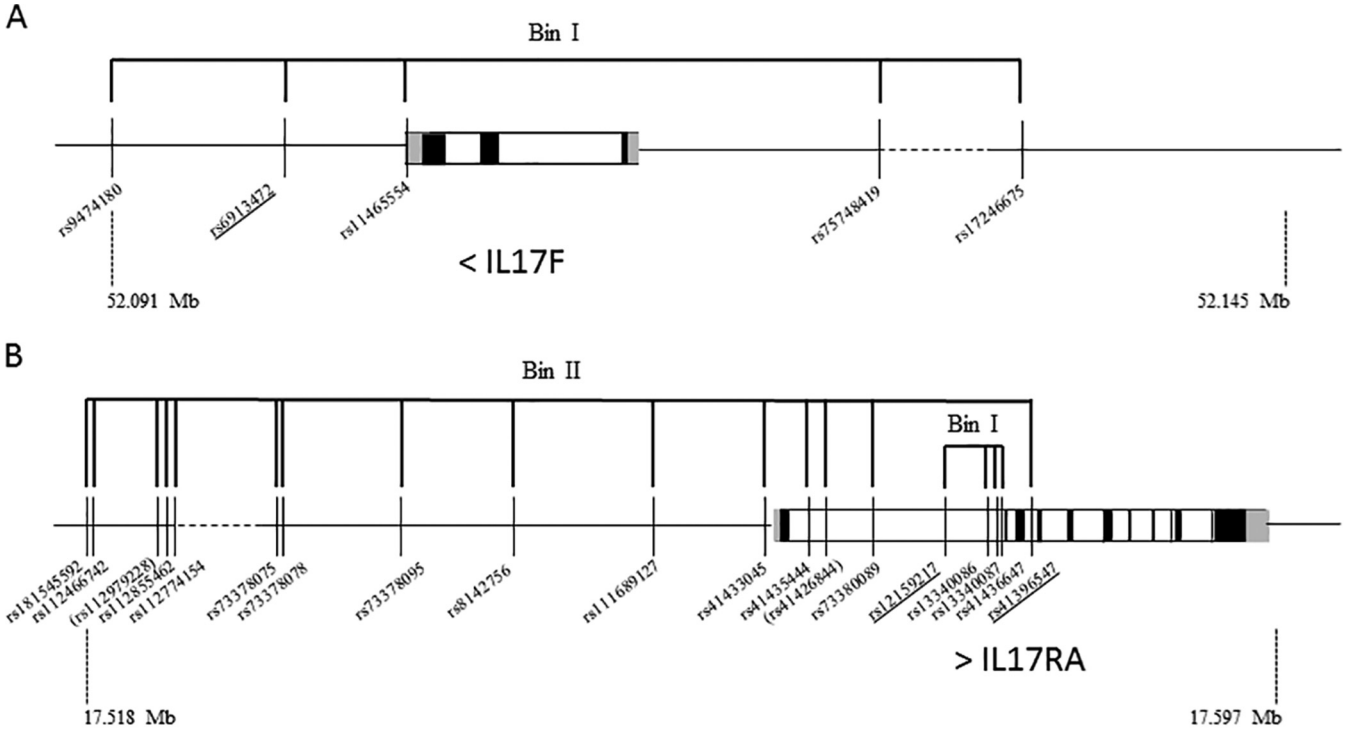

FIG 1 Correlation bins for IL17F and IL17RA genes and flanking regions according to the 1000 Genomes YRI project (18). Pairwise R^2 values from comparisons of SNPs associated with CM in the two study populations (underlined SNPs) and SNPs in a 1-Mb region were determined with PLINK software (19). Polymorphisms belonging to the same correlation group or "bin" ($R^2 > 0.6$) are linked by a thick black line. (A) Chromosomal location of SNPs in the 6p region from 52.091 to 52.145 Mb that includes the IL17F gene (indicated by black vertical lines). The direction of gene transcription is indicated by the "<" sign. Correlation bins with R^2 of >0.6 are as follows: bin I, SNPs rs6913472, rs11465554, rs9474180, rs75748419, and rs17246675. (B) Chromosomal location of SNPs in the 22q region from 17.518 to 17.597 Mb that includes the IL17RA gene (indicated by black vertical lines). The direction of gene transcription is indicated by the ">" sign. Correlation bins with R^2 of >0.6 are as follows: bin I, rs12159217, rs13340087, rs13340086 and rs41436647; bin II, rs41396547 and 14 additional SNPs. The two SNPs whose designations are shown in parentheses, rs112979228 and rs41426844, were not informative and were excluded from the statistical analysis.

stored at −70°C for analysis of cytokine production. Cytokine levels of IL-17F were measured in supernatants as described above. Data are presented as arithmetic means of duplicate values.

Genomic DNA was extracted from peripheral blood leukocytes with a QIAamp blood kit (Qiagen). Genotyping of the *IL17F* rs6913472 SNP was assessed using TaqMan probe assays (Applied Biosystems).

Statistical analysis. A chi-square test was used to determine whether the genotype distributions in parents and in controls conformed to the Hardy-Weinberg equilibrium. None of polymorphisms deviated from the Hardy-Weinberg equilibrium with a significance level of ≤0.05. The analysis was carried out with GenePop software (Web version 4.2, option 1). Univariate and multivariate analyses of SNPs were carried out with SPSS (statistical software version 10.1) to examine the association between SNPs and CM for unrelated Nigerian subjects. Differences were considered significant if the two-sided *P* value was <0.05. We also examined SNP-SNP interactions for independently associated SNPs within the Nigerian population with a two-locus test in PLINK (19). The family-based association test package (FBAT; version 1.7) (20, 21) was used for association tests in nuclear families from Mali. This analysis tests the transmission of the different alleles from heterozygous parents to affected children. A SNP was considered to be replicated if the association analysis yielded a one-tailed *P* value of <0.05 with the same risk allele as in the Nigerian population. A case-pseudocontrol data set analysis and a conditional logistic regression analysis were performed as described previously (16, 22, 23) to estimate odds ratio (OR) values for Malian subjects. Combined analyses that included both the discovery case-control sample and the replication family-based sample were also performed using the conditional logistic regression. Linear regression analysis was performed to test correlations between IL-17F concentrations and *IL17F* rs6913472 genotypes (SPSS statistical software).

***In silico* analysis.** The TFSEARCH program (24) was used to predict the potential transcription factor binding sites in *IL17F* and *IL17RA* genes. Associations of genotype and *IL17F* or *IL17RA* expression were examined with the GeneVar (GENe Expression Variation) database and Java application (25). The gene names and rs identifiers (ID) were entered in the "eQTL-SNP-Gene" option. GeneVar provides Spearman's correlation coefficient and *P* values for analytical comparisons between gene expression and genotype data from 8 different populations. Unfortunately, only the rs6913472 SNP was available.

RESULTS

Several common polymorphisms in *IL17F* and *IL17RA* genes are associated with an increased risk of CM in Nigerian children. We first selected 48 representative TagSNPs within *IL17A*, *IL17F*, and *IL17RA* genes with 5 kb upstream and downstream from these genes. These SNPs were selected from the 1000 Genomes data bank (YRI reference population) (18), and all were common (MAF ≥ 0.05). Forty-six TagSNPs in *IL17A*, *IL17F*, and *IL17RA* were successfully genotyped in the Nigerian samples (see Table S1 in the supplemental material). We found no significant association ($P > 0.05$) between CM and polymorphisms in *IL17A*. In contrast, CM was associated with 3 SNPs in *IL17F*, *IL17F* rs6913472 ($P = 0.039$; OR = 1.95), *IL17F* rs9382083 ($P = 0.004$;

TABLE 2 Results of the population-based association study in the Nigerian cohort among 115 cases with cerebral malaria and 160 healthy controls[a]

Analysis category	Gene	SNP	Position[b]	Minor allele	MAF[c]	Genotype (risk)	% CC cases	% CM cases	OR	95% CI	P^d
Univariate	IL17F	rs6913472[e]	52097072	A	0.10 (0.08)	GA	13.1	22.7	1.95	1.03–3.69	0.039
	IL17F	rs9382083	52097773	A	0.07 (0.10)	GG	74.5	88.5	2.63	1.33–5.18	0.004
	IL17F	rs4715291	52113360	T	0.08 (0.12)	CC	70.0	81.8	1.93	1.07–3.48	0.028
	IL17RA	rs12159217	17573915	T	0.14 (0.16)	GG	64.5	80.0	2.20	1.21–4.02	0.009
	IL17RA	rs41396547	17579054	C	0.04 (0.05)	TT	86.2	94.5	2.76	1.08–7.06	0.028
Multivariate[f]	IL17F	rs6913472		A		GA			3.12	1.43–6.82	0.004
	IL17F	rs4715291		A		GG			2.82	1.39–5.75	0.004
	IL17RA	rs12159217		T		GG			2.27	1.22–4.24	0.010
	IL17RA	rs41396547		C		TT			3.15	1.15–8.70	0.026

[a] Abbreviations: SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; MAF, minor allele frequency. All cases were either cerebral malaria (CM = 112) cases or cerebral malaria and severe malarial anemia (CMSMA = 3) cases. All controls were community controls (CC = 160).
[b] Data represent the position on chromosome 6 for *IL17F* and on chromosome 22 for *IL17RA* according to human hg19 coordinates.
[c] MAF values were estimated from Ensembl for the YRI population according to the 1000 Genomes project guidelines. MAF values for our Nigerian sample are shown in parentheses.
[d] All *P* values represent results of two-sided analyses.
[e] No AA genotype has been observed for rs6913472.
[f] Data represent results of multivariate analysis combining the five significant SNPs (rs6913472, rs9382083, rs4715291, rs12159217 and rs41396547).

OR = 2.63), and *IL17F* rs4715291 ($P = 0.028$; OR = 1.93) (Table 2), and with 2 SNPs in *IL17RA*, *IL17RA* rs12159217 ($P = 0.009$; OR = 2.20) and *IL17RA* rs41396547 ($P = 0.028$; OR = 2.76) (Table 2).

Multivariate analysis stepwise binary association test results confirmed the association of SNPs *IL17F* rs6913472 and *IL17F* rs4715291 as the best model, whereas *IL17F* rs9382083 was excluded. Thus, *IL17F* rs6913472 and *IL17F* rs4715291 were independently associated with CM. Likewise, the results of a multivariate analysis performed on the *IL17RA* polymorphisms indicated that *IL17RA* rs12159217 and *IL17RA* rs41396547 were also independently associated with disease. Finally, the best model revealed by the regression analysis of the SNPs of both genes included *IL17F* rs6913472 ($P = 0.004$; OR = 3.12), *IL17F* rs4715291 ($P = 0.004$; OR = 2.82), *IL17RA* rs12159217 ($P = 0.01$; OR = 2.27), and *IL17RA* rs41396547 ($P = 0.026$; OR = 3.15) (Table 2). Thus, these four SNPs are responsible for the observed associations and exert independent effects on CM. We did not find any evidence for SNP-SNP interactions between these polymorphisms with a two-locus test in PLINK (19).

Comparisons of CM children to UM children from Nigeria revealed evidence of an association between *IL17RA* rs12159217 and CM ($P = 0.037$; OR = 2.04), and a trend of association was obtained with *IL17F* rs6913472 ($P = 0.059$; OR = 2.09) (Table 3). Multivariate analysis combining the two SNPs (rs12159217 and rs6913472) confirmed the association of *IL17RA* rs12159217 with CM ($P = 0.04$; OR = 2.04) (Table 3). Finally, no significant association was detected in comparisons of CC to UM subjects from Nigeria ($P > 0.202$) (Table 3).

***IL17F* and *IL17RA* SNPs are also associated with CM in Malian trios.** We then attempted to confirm these associations of *IL17F* and *IL17RA* SNPs in independent Malian trios. Hence, only the four SNPs significantly associated with CM in the multivariate analysis were selected for genotyping in the familial Malian replication sample. We detected a significant association ($P = 0.03$; OR = 1.39) of *IL17F* rs6913472 with CM (Table 4); the A allele was overtransmitted to children with CM. However, we were not able to replicate the association with rs4715291 in this study population (Table 4). For *IL17RA* polymorphisms, we replicated the same direction of association for rs12159217 ($P = 0.01$; OR = 1.52) and rs41396547 ($P = 0.04$; OR = 3.5). The G allele of rs12159217 was more frequently transmitted to CM children ($n = 127$) than expected from the null hypothesis ($n = 116$), and this association was also significant in permutation testing ($P = 0.01$). For the rs41396547 polymorphism, the same risk allele identified in the Nigerian sample (the T allele) was overtransmitted to children with CM (Table 4).

TABLE 3 Results of the population-based association study in the Nigerian cohort, with cerebral malaria compared to uncomplicated malaria and uncomplicated malaria compared to community controls[a]

Analysis category	Gene	SNP	Genotype (risk)	% CM cases	% UM cases	OR (95% CI)	P^b	% CC cases	% UM cases	P^b
Univariate	IL17F	rs6913472	GA	22.7	12.4	2.09 (1.04–4.17)	0.059	13.1	12.4	1
		rs9382083	GG	88.5	82.6		0.304	74.5	82.6	0.202
		rs4715291	CC	81.8	77.5		0.481	70.0	77.5	0.236
	IL17RA	rs12159217	GG	80.0	66.2	2.04 (1.04–4.00)	0.037	64.5	66.2	0.883
		rs41396547	TT	94.5	92.1		0.570	86.2	92.1	0.217
Multivariate[c]	IL17F	rs6913472	GA			2.09 (1.08–4.74)	0.077			
	IL17RA	rs12159217	GG			2.04 (1.03–4.03)	0.040			

[a] Abbreviations: SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; CC, community controls; UM, uncomplicated malaria; CM, cerebral malaria. All uncomplicated malaria cases were febrile children with *P. falciparum* parasitemia (UM = 89).
[b] All *P* values represent results of two-sided analyses.
[c] Data represent results of multivariate analysis combining the two SNPs (rs6913472 and rs12159217).

TABLE 4 Results of family-based association study in the Malian cohort[a]

Gene	SNP[b]	Minor allele/major allele	Risk allele	Freq[c] (risk allele)	OR (95% CI)	P^d
IL17F	rs6913472	A/G	A	0.109	1.39 (1.13–1.76)	0.03
	rs4715291	T/C	T	0.181		0.17
IL17RA	rs12159217	T/G	G	0.885	1.52 (1.03–2.37)	0.01
	rs41396547	C/T	T	0.976	3.50 (1.06–13.04)	0.04

[a] Abbreviations: SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; Freq, frequency.
[b] Data represent TagSNPs selected for their association with CM in the Nigerian sample and tested for association with CM in the Malian sample.
[c] Risk allele frequency values were estimated from Malian parents.
[d] All *P* values represent results of 1-sided tests.

SNP *IL17RA* rs41433045 in LD with rs41396547 is also associated with CM in the two African populations. To take into account the possibility that additional polymorphisms in LD with associated SNPs may be involved in CM susceptibility, we also included SNPs that correlate ($R^2 > 0.6$) with SNPs *IL17F* rs6913472, *IL17RA* rs12159217, and *IL17RA* rs41396547 on the basis of the data in the 1000 Genomes YRI database (18). Four SNPs in IL17F were correlated in Yoruba with rs6913472 in bin I (Fig. 1A). These SNPs were located close to the 3′ or 5′ end of the *IL17F* gene. Three *IL17RA* SNPs were correlated with rs12159217 in bin I, and 14 SNPs were correlated with rs41396547 in bin II (Fig. 1B). All 17 of these SNPs were located within or close to the *IL17RA* gene in a region of 61 kb. These SNPs were genotyped in the 240 Malian trios. Two of them (rs112979228 and rs41426844) were not informative in our study population and were excluded from the statistical analysis.

No other significant association with SNPs in bin I of *IL17F* and in bin I of *IL17RA* was found. The testing of the 14 SNPs in bin II of *IL17RA* revealed a significant association of rs41433045 ($P = 0.03$ [2-sided test]) with CM that was also significant in the permutation test ($P = 0.04$). The T allele of rs41433045 is overtransmitted to CM children. We thus examined association results between this polymorphism and CM in Nigerian samples, and we confirmed its association ($P = 0.02$) with an OR of developing CM for TT homozygous subjects versus those with a TC genotype, estimated at 3.1 (1.1 to 9.4).

We investigated the LD pattern of the SNPs rs6913472, rs12159217, rs41396547, and rs41433045 in a 1-Mb region, and we found no SNP with an R^2 value of >0.6 in others surrounding genes that could account for the observed associations.

Association results for *IL17F* and *IL17RA* polymorphisms in combined samples. Finally, we performed association comparisons between CM and the 4 SNPs in the whole sample set by combining the genotype data of the Nigerian case-control and the Malian nuclear families. The 3 SNPs of *IL17RA* (rs12159217, rs41396547, and rs41433045) were significantly associated ($P < 0.05$) (Table 5), whereas rs6913472 of *IL17F* indicated a trend of association ($P = 0.059$) (Table 5). The results of multivariate regression analysis performed on the four SNPs showed that *IL17RA* rs12159217 and rs41396547 polymorphisms remained significantly independently associated with CM as follows: for rs12159217, $P = <0.0001$ and OR (95% confidence interval [CI]) = 3.40 (2.22 to 5.21); for rs41396547, $P = <0.0001$ and OR (95% CI) = 4.71 (1.99 to 11.17) (Table 5).

***In silico* analysis of the possible functional effects of the associated SNPs.** Our analysis identified several SNPs within or surrounding *IL17F* and *IL17RA* that were independently associated with CM. *In silico* analysis of these SNPs using TFSEARCH (24) showed that several of them may alter the binding sites of transcription factor: alleles rs6913472 A, rs12159217 T, and rs41433045 C create new binding sites for SRY, Oct-1, and NIT2, respectively. Moreover, the major risk allele, rs12159217 G, created a new binding site for ADR1. Then, we used genotype data from HapMap (26) and gene expression data from GeneVar (25) to perform a quantitative trait locus (eQTL) analysis and assess whether polymorphisms associated with CM are correlated with mRNA abundance. This analysis did not show significant correlations between the transcription levels of *IL17F* rs6913472 genotypes. Data for other SNPs were not available.

Levels of IL-17A and IL-17F were not detectable in plasma. We measured the IL-17A and IL-17F levels by ELISAs of the plasma (diluted by a factor of 2) of 77 children with CM and 45 children with UM from Mali. Levels of IL-17F in the plasma were not detectable in either the CM and the UM children. For IL-17A, the levels were detectable in only 17% of UM and 10% of CM

TABLE 5 Results of combined analyses that included the case-control and familial data[a]

Analysis category	Gene	SNP	Minor allele/major allele	Risk allele	Freq[b] (risk)	OR (95% CI)	P^c
Univariate	IL17F	rs6913472	A/G	A	0.093	1.36 (1.06–1.97)	0.059
	IL17RA	rs12159217	T/G	G	0.882	1.79 (1.24–2.52)	0.001
		rs41396547	C/T	T	0.956	3.17 (1.48–6.78)	0.001
		rs41433045	C/T	T	0.973	4.12 (1.54–11.00)	0.002
Multivariate[d]	IL17RA	rs12159217	T/G	G	0.882	3.40 (2.22–5.21)	<0.0001
		rs41396547	C/T	T	0.956	4.71 (1.99–11.17)	<0.0001

[a] Abbreviations: SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; Freq, frequency.
[b] Data represent risk allele frequencies.
[c] All *P* values represent the results of 1-sided tests in reference to the risk allele.
[d] Data represent results of multivariate analysis combining the four SNPs (rs6913472, rs12159217, rs41396547, and rs41433045).

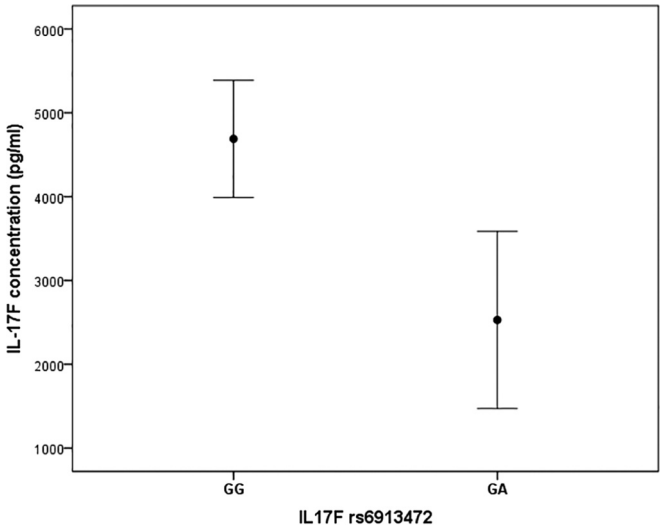

FIG 2 Concentration of IL-17F according to the *IL17F* rs6913472 genotypes. The GA genotype was associated ($P = 0.02$) with the lower levels of IL-17F production in supernatants from the cell cultures of 50 healthy subjects. The concentrations are represented as arithmetic means of duplicate values (± standard errors of the mean).

children. Hence, the number of subjects with detectable IL-17A levels was too low to perform statistical analysis.

Aggravating genotype *IL17F* rs6913472 GA was correlated with lower IL-17F concentration. To further explore the potential functional effect of the rs6913472 polymorphism, we sought to determine whether genotypes may be correlated with the level of the IL-17F protein. Hence, we analyzed the IL-17F production in cultures of PBMCs from healthy subjects from the Marseille blood bank stimulated with anti-CD3/anti-CD28 antibodies. We found that the at-risk GA genotype of rs6913472 was significantly associated with lower levels of IL-17F production ($P = 0.02$) (Fig. 2).

DISCUSSION

The aim of this study was to evaluate whether genetic variants in IL-17A and IL-17F influence a child's risk of developing cerebral malaria. We thoroughly analyzed genetic variants of *IL17A* and *IL17F* and of their common receptor, *IL17RA*, in two independent African populations. We found that CM was significantly associated with SNPs in *IL17F* and *IL17RA* in a population of children from Nigeria. We then tested these findings in a cohort of nuclear families from Mali.

Among polymorphisms in *IL17F*, the rs6913472 and rs4715291 SNPs showed the strongest association with CM in Nigerian children and rs6913472 was replicated in the Malian sample. The rs12159217 polymorphism of the *IL17RA* gene was associated with CM in both the Nigerian and Malian populations ($P < 0.05$ in both cohorts) with the same G risk allele. Similarly, *IL17RA* rs41396547 and rs41433045 (in strong LD; $R^2 = 0.67$, $D' = 1$) were associated in both populations with the major T allele as a risk for CM. Our findings showed that all SNPs associated with CM are located near or within the *IL17F* or *IL17RA* genes. The regional LD patterns indicated that no SNPs in the 1-Mb region and in strong LD with the associated SNPs are located in genes other than *IL17F* and *IL17RA*, supporting the idea of the involvement of these genes in the development of CM.

The association observed between *IL17F* rs4715291 and CM in the Nigerian sample was not replicated in the Malian population. This SNP is in strong LD ($R^2 = 0.76$) with the nonsynonymous SNP rs763780, which did not show convincing results in our two study populations. The rs763780 was previously described as a functional polymorphism (27) and has found to be associated with susceptibility to tuberculosis (28) and resistance to asthma (27). This result may have been due to the poor conservation of LD blocks among African populations, which is widely recognized. Indeed, this may explain in part the limited success of studies carried out with a pool of samples from various African ethnic groups, because microarrays include TagSNPs that have been defined in a few African populations. This problem occurs less frequently in other world populations (i.e., European, Asian, and American populations) that diverged much later than African populations. This underlines the importance of carefully determining LD patterns before attempting validation studies in different African populations.

Using TFSEARCH (24), which provides an *in silico* prediction model for transcription factor binding, we found that transcription factors bound to the DNA sequence overlapping several associated SNPs (rs6913472, rs12159217, and rs41433045) when a particular allele was present. These results indicate that these SNPs may play a functional role by acting on regulatory functions. Unfortunately, plasma levels of IL-17F were not detectable in either UM and CM Malian subjects. Hence, no correlation could be established between the genotype and the levels of IL-17F. We thus performed functional studies to investigate the potential functional effect of the *IL17F* rs6913472 polymorphism on cultures of PBMCs. The rs6913472 GA genotype was associated with a lower level of IL-17F production in anti-CD3/CD28-stimulated cells. Thus, rs6913472 GA is associated with (i) downregulation of IL-17F, (ii) modification of transcription factor binding, and (iii) an increased risk of CM.

IL-17F stimulates the production of both antimicrobial peptides and molecules capable of recruiting or stimulating immune cells (granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-6) and chemokines such as CXCL1, CXCL2, and CXCL5 that promote neutrophil recruitment. Both IL-17A and IL-17F stimulate matrix metalloproteases, thus increasing cell mobility. Studies in mice showed that IL-17A, IL-17E, IL-17F, and IL17RA are required for host resistance to *Trypanosoma cruzi* (29); furthermore, human resistance to kala azar caused by *Leishmania donovani* was associated with high levels of IL-17 and IL-22 responses (30). IL-17A and IL-17F are also crucial in the clearance of extracellular bacteria, such as *Staphylococcus aureus*, *Citrobacter rodentium*, and *Klebsiella pneumoniae* (31, 32).

However, several studies indicate that Th17 cells aggravate neurological disorders such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) (7). The pathophysiology of MS involves a neuroinflammatory reaction and disruption of the blood-brain barrier (BBB). These pathophysiological changes are also found in CM, suggesting that Th17 cells may play a similar role in the development of cerebral manifestations associated with *P. falciparum*. Cytokines secreted by Th17 cells, including IL-17 and IL-22, may alter the permeability of the human BBB to soluble molecules and circulating $CD4^+$ lymphocytes (33). IL-17A is also required for the initiation of EAE, whereas IL-17F plays only a minor role and maintains in-

flammation in the central nervous system (CNS) (34). Indeed, anti-IL-17F treatment does not improve the course of EAE whereas the onset of EAE is delayed and its progression is slow in IL-17 knockout (KO) mice and wild-type mice treated with anti-IL-17 (35, 36). Our data, however, do not support the hypothesis that IL-17A and IL-17F could aggravate CM by increasing inflammation in particular because (i) we found no association between CM and SNPs in *IL17A*, whose product is more proinflammatory than IL-17F, and (ii) subjects with the at-risk GA genotype associated with *IL17F* rs6913472 had significantly lower IL-17F production than genotype GG subjects.

In the experimental CM mouse model infected with *P. berghei ANKA* (PbA) (sharing certain characteristics with human CM), mice deficient in IL-23 or IL-17A develop neurological symptoms and die, similarly to wild-type mice (37), suggesting that these cytokines do not aggravate CM. Thus, in spite of the observation that IL-17A aggravates inflammation associated with various neurophysiological disorders, our data from children and observations in mice do not support the view that IL-17A increases the risk of CM. In fact, we suggest that the presence of IL-17A and IL-17F increases antiparasite immunity during infection by *P. falciparum* and then protects against severe disease.

All IL-17 family members use receptors that share the common IL-17RA chain. IL-17A and IL-17F signal through IL-17RA or through the dimeric receptor IL-17RA/IL-17RC (38); however, these two cytokines interact differently with the two chains. IL-17F is highly dependent on IL-17RC, whereas IL-17A can signal through IL-17RA alone (39, 40). The expression of IL-17RC is restricted to nonhematopoietic tissue-resident cells, suggesting that the selective action of IL-17F on nonhematopoietic cells may be due to the presence of IL-17RC. IL-17RA is widely expressed by innate cells such as macrophages and neutrophils and by nonhematopoietic cells such as epithelial cells and fibroblasts (38). Our finding that polymorphisms in *IL17RA* are associated with CM confirms that cytokines belonging to the IL-17 cytokine family are implicated in malaria. Given that IL-17F relies on IL-17RC more than on any other IL-17-related cytokine, we are currently examining genetic variants of this receptor gene and their associations with CM.

In conclusion, we used discovery and replication cohorts of childhood *P. falciparum* malaria to show that polymorphisms in *IL17F* and *IL17RA* significantly contribute to susceptibility to CM. Functional studies provide evidence that lower production of IL-17F leads to CM. Further investigation of this promising association is required to decipher the underlying mechanisms of the involvement of these genes in the pathogenesis of CM. Finally, our validated findings expand current knowledge of the complex host genetic factors that predispose to childhood cerebral malaria. Our results are relevant for the development of diagnostic algorithms to identify children who have a high risk of developing CM. Furthermore, such diagnostic algorithms will also guide the design of functional studies aimed at the development and deployment of severe malaria prevention strategies. Our results emphasize the importance of deciphering the role of this key immunological pathway in the pathogenesis of severe childhood malaria. More importantly, our results may contribute to the development of adjunct therapies aimed at reducing mortality and neurological sequelae in children presenting with cerebral malaria.

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